CHEMBIOCHEM

DOI: 10.1002/cbic.200500075

Synthesis of Glycopeptides from Type II Collagen-Incorporating Galactosylated Hydroxylysine Mimetics and Their Use in Studying the Fine Specificity of Arthritogenic T Cells

Julien Marin,[a] Marie-Agnès Blaton,[b] Jean-Paul Briand,[a] Gilles Chiocchia,[b] Catherine Fournier,*[b] and Gilles Guichard*[a]

*Five analogues of the bovine type II collagen (bCII) immunodominant glycopeptide [β-D-Gal-(5R)-5-Hyl264]CII(256–270) (**1**) carrying diverse modifications at the critical hydroxylysine (Hyl) 264 side chain were designed and synthesised, to explore the fine specificity of bCII-reactive T cells involved in the initiation and/or regulation of collagen-induced arthritis (CIA), a mouse model for rheumatoid arthritis (RA). β-D-Galactosyl-(5R)-5-hydroxy-L-lysine (**19**) and corresponding mimetics (**22**–**25**), conveniently protected for solid-phase synthesis, were all obtained by a divergent route involving enantiopure 5-hydroxylated 6-oxo-1,2-piperidinedicarboxylates as the key intermediates. All three bCII-specific T hybridomas used, as well as a recurrent pathogenic CD4$^+$ T-cell clone isolated from bCII-immunised DBA/1 mice, recognised the galactosylated form **1** of the immunodominant bCII (256–270) epitope. These cells were extremely sensitive to changes at the ε-amino group of Hyl264, but differed in their pattern of recognition of analogues with a Hyl264 side chain modified at C-5 (i.e. inversion of stereochemistry, methylation). These data further document the importance of collagen post-translational modifications in autoimmunity and in the CIA model in particular, and provide a new insight into the molecular interaction between glycopeptide **1** and the TCR of pathogenic T cells.*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the distal joints that leads to cartilage destruction and bone erosion. RA is one of the most frequent systemic autoimmune diseases and affects a large proportion of the world's population (prevalence between 0.3 and 1% in industrialised countries and an overall prevalence of 0.8% of adults).[1] Women, in whom the condition most frequently starts at menopause, are three times more likely to be affected by RA than men. However, in about 25% of cases, the disease appears before the age of 40 and sometimes even during childhood (juvenile chronic arthritis). This articular pathology is associated with a massive infiltration of leukocytes (i.e., T cells and other immune cells including B cells, macrophages and mast cells), which, together with activated synoviocytes, form the aggressive front of proliferative tissue (pannus) that overgrows the articular cartilage.

The link between RA and genes of the class II MHC molecules (80% of Caucasian patients with RA express DR4 or DR1 allotypes), as well as the large number of activated CD4$^+$ T cells in arthritic joints, support a central role for T cells in the pathogenesis of RA.[2] Type II collagen (CII), the major protein found in joint cartilage, has been proposed as a candidate autoantigen in RA.[3] A direct role for collagen in the development of arthritis has been demonstrated in collagen-induced arthritis (CIA),[4] which presents most of the clinical, histological and immunological characteristi'cs of RA.[5] Like RA, susceptibility to CIA is strongly linked to MHC class II haplotypes and, in mice, it is associated with strains expressing A^q and A^r molecules. The peptide CII(256–270) has been identified as an immunodominant T-cell epitope on CII, Ile260 and Phe263 serving as primary anchor residues for binding to the MHC class II A^q molecule.[6] It is worth noting that humanised mice transgenic for DR4 and DR1 are also susceptible to CIA, and, in this case, the sequence of the immunodominant T-cell epitope is

[a] *Dr. J. Marin, Dr. J.-P. Briand, Dr. G. Guichard*
UPR 9021 CNRS—Immunologie et Chimie Thérapeutiques (ICT)
Institut de Biologie Moléculaire et Cellulaire
15 rue René Descartes, 67084 Strasbourg Cedex (France)
Fax: (+33) 388-610-680
E-mail: g.guichard@ibmc.u-strasbg.fr

[b] *M.-A. Blaton, Dr. G. Chiocchia, Dr. C. Fournier*
Département d'Immunologie
Institut Cochin, INSERM U567, CNRS UMR 8104, Université Paris 5
Pavillon Hardy A
27 rue du Faubourg-Saint-Jacques, 75674 Paris Cedex 14 (France)
Fax: (+33) 143-261-156
E-mail: fournier@cochin.inserm.fr

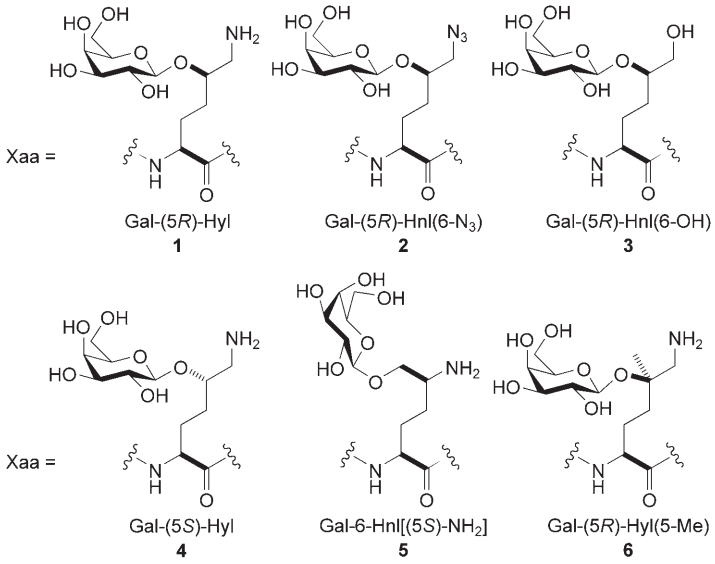
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

only slightly shifted and encompasses residues 261–273 (CII-(261–273)).[7] Holmdahl and Kihlberg recently recognised the importance of post-translational modifications of CII in generating immunogenic self-determinants relevant to the development of CIA and RA. In particular, they demonstrated that CIA and RA T cells predominantly recognise glycosylated forms of immunodominant CII peptides with a Gal-5Hyl side chain at position 264 serving as critical T-cell receptor (TCR) contact.[8]

We previously identified CII-reactive T cells in A^q mice immunised with bovine CII (bCII) that participate in CIA course and regulation.[9,10] To study their fine specificity, we envisaged a strategy based on alteration of the Gal-5-Hyl264 T-cell-recognition motif through slight changes in the 5-Hyl side-chain. Eventually, structural modulations of the TCR contacts might also lead to the generation of altered peptide ligands (APLs)[11] useful for manipulating the immune response in affected joints. It is now well established that TCR engagement by antigens containing minor modifications at positions that contact the TCR can result in various functional outcomes including T-cell partial agonism and antagonism, anergy and apoptosis. The potential of APLs as modulators of autoimmune diseases and allergies through immune deviation has been successfully demonstrated in several animal models (e.g. experimental autoimmune encephalomyelitis[11bj]). Here, we describe the synthesis and in vitro immunological evaluation of the known immunodominant peptide [Gal-5-Hyl264]bCII(256–270) (**1**) and several naturally occurring variants, together with five original glycopeptide analogues (**2**–**6**) modified at the Hyl side chain (Scheme 1).

Glycopeptides **2** and **3,** in which the N^{ε}-amine is replaced by a hydrophobic azide function and by a hydrophilic hydroxyl group, respectively, were synthesised to evaluate the permissiveness of the N^{ε}-amino group in interaction with T cells. The plasticity of the T-cell-recognition motif (galactose and N^{ε} primary amine) at position 264 was investigated through several other modifications of the Hyl side chain: inversion of stereochemistry at C-5 (→**4**), permutation of the N^{ε}-amino and carbohydrate moieties (→**5**) and the introduction of steric congestion at C-5 (→**6**).

H-Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-**Xaa264**-Gly-Glu-Gln-Gly-Pro-Lys270-OH

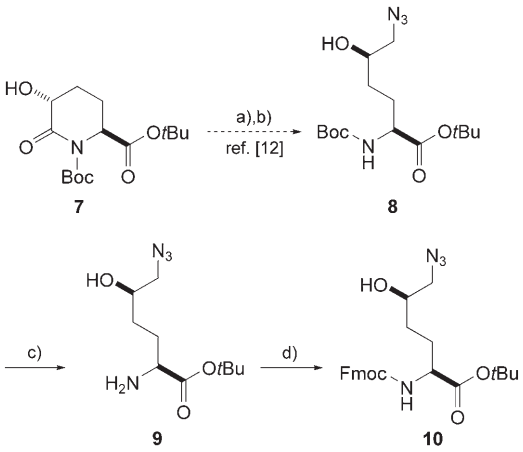


Scheme 1. List of the target glycopeptides.

Results and Discussion

Aglycons synthesis

Our general approach to the synthesis of conveniently protected (2*S*,5*R*)-5-Hyl for the synthesis of **1** and **2** is based on the use of α-monohydroxylated δ-lactam **7** as a key intermediate (Scheme 2).[12]



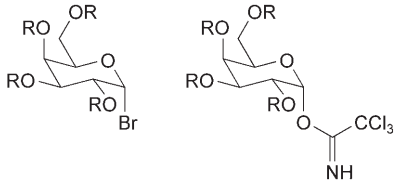
Scheme 2. Preparation of the orthogonally protected (2*S*,5*R*)-5-hydroxylysine derivative **10**. a) $NaBH_4$, EtOH, (91%); b) i) MsCl, collidine, CH_2Cl_2; ii) NaN_3, DMF, (88%); c) PTSA, CH_3CN, 0°C→RT, then NH_4OH, (99%); d) FmocOSu, $NaHCO_3$, THF/H_2O (77%).

For convenient solid-phase peptide synthesis (SPPS), N^{α}- and N^{ε}-amino functionalities were protected by a Fmoc group and as an azide, respectively. Aglycon **10** was obtained from the 1,2-azidoalcohol **8**[12] in a two-step procedure. Various known methods[13] for the selective cleavage of the *N*-Boc protecting group in the presence of the *tert*-butyl ester were investigated. The use of *p*-toluenesulfonic acid (PTSA) in acetonitrile[13c] was the sole satisfactory procedure for **8**. This selective deprotection reaction required careful TLC monitoring, because the kinetics and the equilibrium of Boc deprotection versus *tert*-butyl ester were very sensitive to both time and temperature. Neutralisation with NH_4OH afforded free amine **9**, which was directly converted into the *N*-Fmoc derivative **10**. The same overall strategy was used for the synthesis of related amino acid aglycons required for the preparation of peptides **3**–**6**.[14]

Synthesis of galactosylated building blocks

As briefly stated previously[14], the use of tetra-pivaloylated galactosylated donors is required for successful galactosylation of protected (2*S*,5*R*)-5-Hyl

aglycon **10**.[15,16] Koenigs–Knorr[17] and Schmidt's trichloroacetimidate[18] procedures with tetra-acylated galactosylated donors resulted exclusively in the formation of either the corresponding orthoester, the rearranged acetylated 5-hydroxylysine or a mixture of both (see supporting information). The formation of orthoester was completely suppressed by using pivaloylated galactosyl donors **11** or **12** (Table 1).

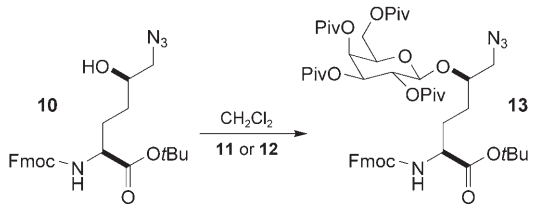


11 R = Piv **12** R = Piv

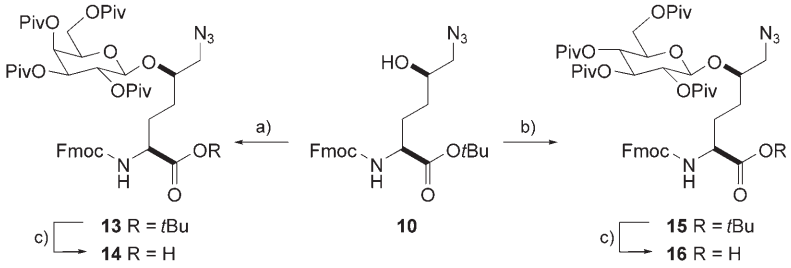
Under optimised conditions (entry 2), the galactosylated derivative **13** was obtained in 83% yield; this clean reaction being easily monitored by C_{18} RP-HPLC. Acid-catalysed cleavage of the *tert*-butyl ester quantitatively gave building block **14** ready for use in SPPS (Scheme 3). Alternatively, glycosylation with tetrapivaloylated glucosyl bromide yielded glucosylated (2*S*,5*R*)-5-hydroxylysine **15** in 76% (Scheme 3).

Galactosylation of 5-hydroxylysine mimetics with tetrapivaloylated galactosyl bromide in the presence of silver silicate under very similar conditions afforded the glycosylated building blocks **17**–**20** required for the preparation of non-natural glycopeptides **3**–**6**, in yields ranging from 62 to 89% (Scheme 4).[14]

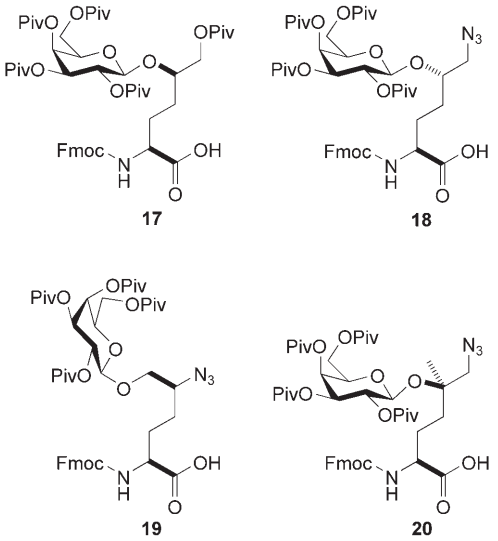
Table 1. Donor and promoter study with pivaloylated galactosyl donors.



	Donor	Lewis acid	*T* [°C]	*t* [h]	Yield [%]
1	**11**	silver silicate	RT	72	62
2	**11**	silver silicate	RT	16	83
3	**12**	$Et_2O{\cdot}BF_3$	RT	24	46
4	**12**	TMSOTf	−10	0.5	64



Scheme 3. Synthesis of the glycosylated (2*S*,5*R*)-5-hydroxylysine building blocks **14** and **16**. a) silver silicate, Gal(Piv)$_4$Br, CH_2Cl_2 (83%); b) silver silicate, Glc(Piv)$_4$Br, CH_2Cl_2 (76%); c) TFA/CH_2Cl_2, quant.



Scheme 4. Galactosylated building blocks **17**–**20** were obtained by galactosylation of the corresponding aglycon (see ref. [14]) with **11** under the promotion of silver silicate in yields ranging from 62 to 89%.

Glycopeptide synthesis

With *N*-Fmoc-protected glycosylated building blocks **14** and **16**–**20** in hand, we prepared the corresponding glycopeptides **1**–**6** derived from CII(256–270) and [Glc-5-Hyl264]bCII(256–270) (**21**) using conventional solid-phase peptide-synthesis procedures (Table 2).[19] Several naturally occurring CII(256–270) peptides (**22**–**25**) bearing various degrees of post-translational modifications were also prepared (Table 2). Peptide **22** differs from **1** at position 258 (Pro versus Hyp). Peptide **23** is the mouse (m) CII analogue of glycopeptide **1** (homologous peptide) and bears an Asp residue at position 266. Peptides **24** and **25** are the nonglycosylated analogues of **1** and **22**, respectively.

All glycopeptides were assembled on polystyrene Wang resin[20] (60 μmol scale) by using a home-made peptide synthesiser.[21] For **1**, peptide purity after elongation of the peptide chain and prior to azide reduction on solid support was checked by cleavage of an aliquot of peptide resin and found to be >90% (as determined by C_{18} RP-HPLC, see Figure 1a). Treatment of the resin with either trimethylphosphine (PMe_3) or triphenylphosphine (PPh_3) in THF resulted in clean and quantitative reduction of the azido function after 72 h, as monitored by RP-HPLC. Cleavage from the resin was performed with TFA containing water and scavengers. At this stage of the synthesis, the purity of the precursor of **1** bearing a protected galactosyl moiety (**26**) was 87% (Figure 1b). An additional step was necessary to remove the pivaloyl ester protecting groups. A diluted solution of NaOMe in MeOH (40 mM) was used to avoid epimerisation of the amino acid α-stereogenic centres along the peptidic chain. Monitoring the reaction by C_{18} RP-HPLC revealed a complete cleavage of all four pivaloyl groups after 8–12 h, without significant formation of degradation by-products. At this stage of the synthesis, the

Table 2. Sequence and analytical data for all CII-derived peptides and glycopeptides. H-Gly-Glu-**Xaa258**-Gly-Ile-Ala-Gly-Phe-**Xaa264**-Gly-**Xaa266**-Gln-Gly-Pro-**Lys270**-OH

Peptide	Xaa258	Xaa264	Requested building block for Xaa264	Xaa266	Calc. Mass	MS found [$M+H^+$]	Yield [%]	Purity [%] HPLC
1	Hyp	Gal-(5*R*)-Hyl	**14**	Glu	1665.75	1667.11	59	>99
2	Hyp	Gal-(5*R*)-Hnl(6-N_3)	**14**	Glu	1691.75	1693.02	59	>99
3	Hyp	Gal-(5*R*)-Hnl(6-OH)	**17**	Glu	1666.74	1667.01	58	>99
4	Hyp	Gal-(5*S*)-Hyl	**18**	Glu	1665.75	1667.84	58	95
5	Hyp	Gal-6-Hnl[(5*S*)-NH_2]	**19**	Glu	1665.75	1666.70	29	>99
6	Hyp	Gal-(5*R*)-Hyl(5-Me)	**20**	Glu	1679.78	1680.40	57	>99
21	Hyp	Glc-(5*R*)-Hyl	**16**	Glu	1665.75	1667.28	55	95
22	Pro	Gal-(5*R*)-Hyl	**14**	Glu	1649.75	1651.18	56	99
23	Hyp	Gal-(5*R*)-Hyl	**14**	Asp	1651.73	1653.66	58	>99
24	Hyp	Lys	–	Glu	1487.61	1488.85	95	98
25	Pro	Lys	–	Glu	1471.61	1473.08	95	>99
26	Hyp	Gal(Piv_4)-(5*R*)-Hyl	**14**	Glu	2002.22	2002.65	73	>99

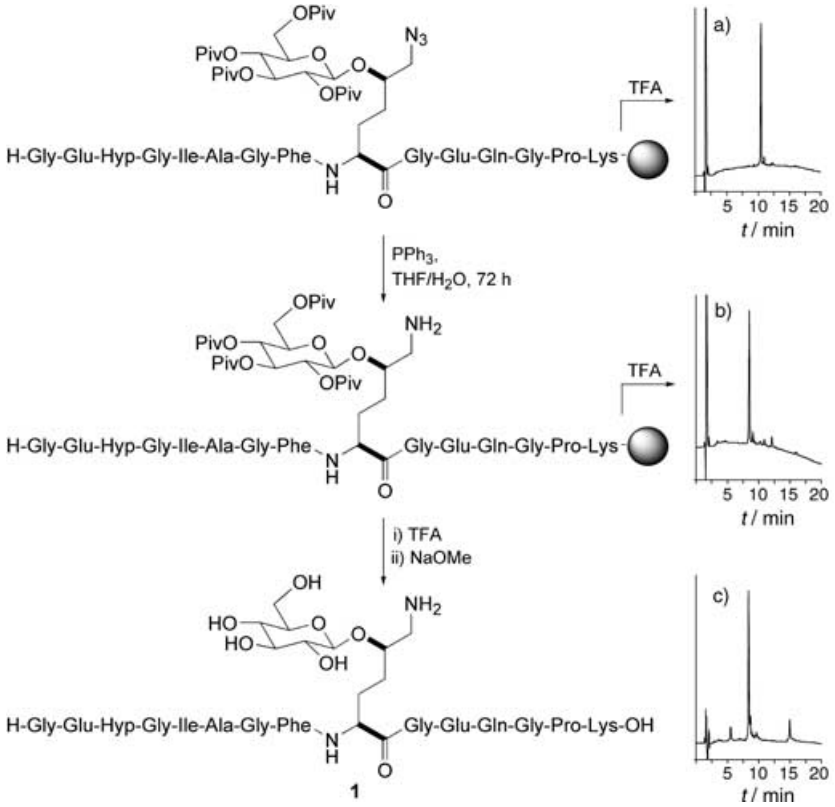


Figure 1. C_{18} RP-HPLC profiles of crude peptide intermediates at different stages of the synthesis of **1**.

purity of the naturally occurring glycopeptide **1** was 71% (Figure 1c). The purities of crude glycopeptides **2**–**4** and **6** were in the same range. In the case of **5**, however, cleavage from the resin after reduction of the azido group gave a 1:1 (*w*/*w*) mixture of the expected crude pivaloylated glycopeptide ([$M+H^+$] = 2001.98) and a major impurity **27,** which was subsequently found to be the corresponding alkylaminotriphenylphosphonium salt derivative ([$M+H^+$] = 2262.26) resulting from incomplete hydrolysis of the intermediate iminophosphorane.[22]

Finally, CII-derived glycopeptides **1**–**6**, **21**–**23** and **26** were purified by RP-HPLC and recovered in fair to good overall yields (29–73%), based on resin loading (Table 2). All peptides were identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS), and their homogeneity was assessed by C_{18} RP-HPLC (purity of all peptides determined to be >99%).

T-cell recognition of naturally occurring CII(256–270) peptides

In the course of investigating T cell responses in CIA developing in DBA/1 (A^q) mice, we generated three bCII-specific T hybridomas (A8E2, A2G10 and A9E5) that were able to modulate the disease following a T-cell vaccination protocol.[9] Several years later, a recurrent pathogenic $CD4^+$ T-cell clone (named A9.2) was isolated from bCII-immunised DBA/1 mice.[10] Interestingly, the hybridomas and the T-cell clone expressed closely related TCRs and recognised glycosylated peptides comprising the immunodominant bCII (256–270) epitope. To elucidate the fine specificity of these cells, we first tested the stimulating capacity of the naturally occurring synthetic peptides from bCII that we prepared. Regardless of which T cells were used, galactosylation of Hyl264 (carried by peptides **1** and **22**) was required to trigger T cell reactivity, since nonglycosylated analogues (peptides **24** and **25**) had no effect, even at high peptide concentrations (Figure 2). Glycopeptides **1** and **22**, varying only at residue 258 (Hyp vs. Pro), were recognised by all T cells; this indicates that post-translational hydroxylation of Pro258 is not essential for T-cell recognition. Moreover, the mutation

Glu266Asp (**1** versus **23**), which corresponds to the autologous mCII sequence, only marginally influences the T cell responses (Figure 2).

T-cell recognition of chemically modified glycopeptides

To further define the epitope specificity of the T cells reactive to peptide **1**, we used several analogues modified at the Hyl side chain (Scheme 1). CII-derived T cells were extremely sensitive to modification of the ε primary amine, notwithstanding the presence of the galactosyl group. Though a weak reactivity at high concentrations could be observed sometimes, the azido derivative **2** was hardly recognised by the three CII-specific T-cell hybridomas tested, nor by the T-cell clone (Figure 3). Similarly, peptide **3** with a $^{\varepsilon}$OH group did not activate any of the CII-specific T cells (Figure 3). To ensure that peptides **2** and **3** could still bind to MHC molecules, we checked their in vivo immunogenicity in mice primed with the analogues emulsified in CFA (data not shown). This is consistent with previously published data showing that the A^q-binding capacity of the galactosylated CII 256–270 immunodominant epitope is not affected by deamination of the Hyl side chain (i.e., replacement of hydroxylysine by norvaline).[8b]

These results further highlight the importance of the N^{ε}-amino group for recognition by T cells[8c] and indicate the lack of permissiveness at this position. The protonated ε-amine of the Hyl264 side chain is likely to create crucial contacts with polar residues of the TCR surface. However, another hypothesis might be considered; namely, the participation of the amine in the formation of an intramolecular salt bridge with the Glu266 side-chain, thus positioning and stabilising the galactosyl moiety for T-cell recognition.

Not surprisingly, peptide **26**, which carries a fully protected galactosyl moiety, did not induce T-cell activation (data not shown). Also, in line with a previous report by Holm et al.,[8e] glycopeptide **21**, which has a glucosyl (instead of galactosyl) moiety, failed to stimulate two of the T hybridomas, as well as the A9.2 clone (data not shown). This finding emphasises the requirement for an axial HO-4 group on the carbohydrate molecule for T cell responses.

Comparison of the proliferation indexes obtained in the presence of peptides **1** and **4** revealed the importance of the stereochemistry at C-5 of the GalHyl264 residue (Figure 3). Although the inversion of configuration at C-5 was found to be partially detrimental for stimulation of A2G10 and A9E5 hybridoma, peptide **4** was nevertheless recognised by A8E2 hybridoma, albeit at higher concentrations than peptide **1**. Thus, provided that the required pharmacophores, namely the HO-4 group of the galactosyl res-

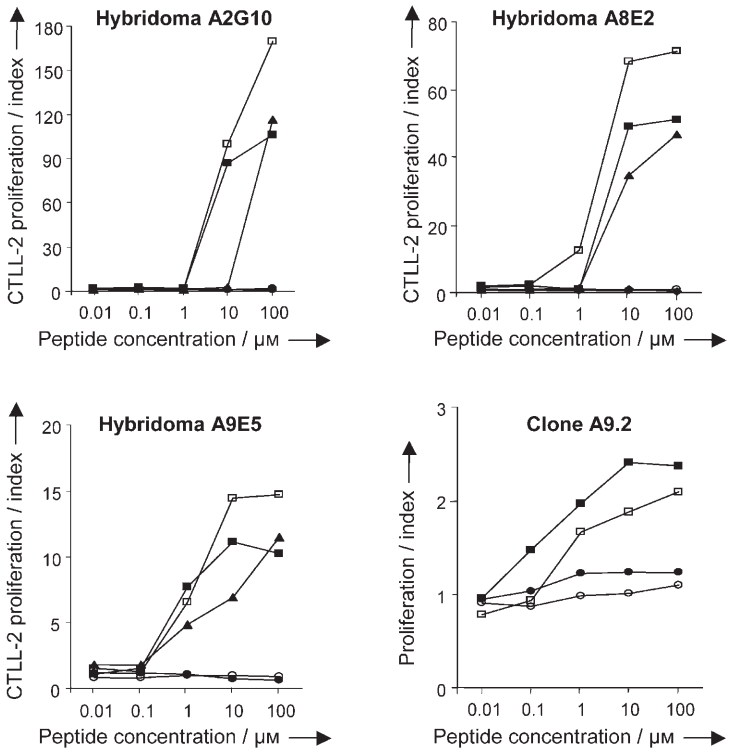


Figure 2. Recognition of naturally occurring CII(256–270) peptides by CII-specific T cells. The anti-CII T hybridomas and clone expressing TCR with closely related structure were incubated with varying concentrations of the indicated peptides. Their reactivity was determined by measuring proliferation for the A9.2 clone and IL-2 secretion (based on proliferation of IL-2 sensitive CTLL-2 cell line) for the hybridomas, as detailed in the Experimental Section. Data are mean values of 2–4 individual experiments. Peptides: ■ = **1**, □ = **22**, ▲ = **23**, ● = **24**, ○ = **25**.

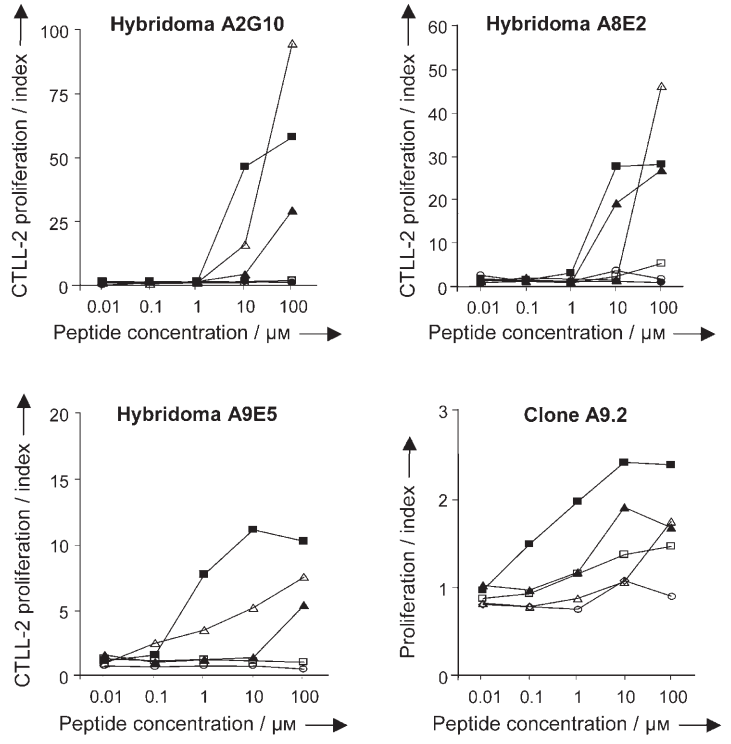


Figure 3. T-cell reactivity to glycopeptides with GalHyl264 derivatives modified at the ε-primary amine (**2**,**3**) and at C-5 (**4**–**6**) compared to **1**. For experimental procedures, see legend of Figure 2. Peptides: ■ = **1**, □ = **2**, ○ = **3**, ▲ = **4**, ● = **5**, △ = **6**.

idue and the primary amino group at the ε position are present, slight changes in their relative orientation and/or position are partially tolerated. This is confirmed by the results obtained with **6** bearing a disubstituted C-5 carbon (i.e., hydroxylated and methylated). Like **1**, **6** is recognised by the three hybridomas (Figure 3). Although this modification creates some steric congestion in the vicinity of the galactosyl moiety and the ε-primary amino group, it is tolerated by the TCR which, in this case, demonstrates some plasticity. However, permutation of the carbohydrate and amino groups to give the glycopeptide **5** was found to be fully detrimental to recognition by T cells.

Conclusion

Taken together, the results obtained with the "naturally occurring" or "chemically modified" CII glycopeptides give information about the relative position of the elements composing the TCR recognition pattern. Interestingly, even if key elements have been identified for the interaction with the TCR (i.e., the ε-primary amine and the HO-4 of the galactosyl moiety), their position relative to each other in the epitope does not necessarily have to be frozen in order to generate a T cell response. It appears that the ternary interaction shows some plasticity, since both the inversion of stereochemistry and the introduction of a methyl group at C-5 are permitted to a large extent.

The protected β-D-galactosyl-(5*R*)-5-hydroxy-L-lysine building block **14** required for the synthesis of natural glycopeptide [β-D-Gal-(5*R*)-5-Hyl264]CII(256–270) **1** was obtained in only 11 steps and in 36% yield from Boc-Asp-O*t*Bu, a readily available starting material. The same efficient, modular approach featuring α-hydroxylated δ-lactams as key intermediates and pivaloylated galactosyl bromide as a galactosyl donor was employed to access the galactosylated hydroxylysine mimetics **17**–**20** required for the synthesis of all new analogues of **1** modified at the Hyl side chain.

There is increasing evidence from the literature that post-translational modifications of antigens and particularly glycosylation play a major role in autoimmune diseases.[23] In CIA, one of the mouse models most relevant for rheumatoid arthritis, the arthritogenicity of CII depends on its carbohydrate content.[8a] Very recently, it was reported that, in healthy joint cartilage, the major immunodominant CII (256–270) T epitope is glycosylated at position 264, whereas, in the arthritic one, both glycosylated and non-glycosylated CII forms are present.[24] These findings suggest that partial CII deglycosylation could be indicative of arthritis progression and further highlight the unique role played by T cells specific for post-translational modification at different stages of disease development. Since all the cells used in this study were shown to participate in CIA initiation and/or regulation, they represent appropriate tools for understanding, at the molecular level, the law governing the recognition of CII carbohydrate contents by pathogenic T cells during CIA course. In agreement with other studies,[25] we highlight here the recognition of intact galactose moiety carried by Hyl264 within the immunodominant CII (256–270) epitope in triggering the pathogenic TCR. In addition, using multiple original analogues of the natural peptide, we further documented the crucial molecular interaction between the TCR and its cognate peptide, as well as the conformational structure requirement for T-cell activation. Such findings will contribute to elaborating highly specific peptide therapy with altered peptide ligands.[11,26]

Experimental Section

Materials: Galactosyl donors **11** and **12** were prepared according to standard procedures, starting from commercial pentapivaloylated D-galactose.[27] Silver silicate was prepared according to a reported procedure[28] and dried at 110 °C under high vacuum for 6 h before use.

General methods: THF was distilled from Na/benzophenone. CH_2Cl_2 and cyclohexane were distilled from CaH_2. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck) with detection by UV light and charring with 1% (*w/v*) ninhydrin in ethanol followed by heating. Flash column chromatography was carried out on silica gel (0.063–0.200 nm). HPLC analysis was performed on a Nucleosil C_{18} column (5 μm, 3.9×150 mm) by using a linear gradient of A (0.1% TFA in H_2O) and B (0.08% TFA in CH_3CN) at a flow rate of 1.2 mL min^{-1} with UV detection at 214 nm. Optical rotations were recorded with a Perkin–Elmer polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance apparatus. Mass spectra were recorded by using a MALDI-TOF apparatus (Bruker Protein-TOF).

***tert*-Butyl (2*S*,5*R*)-6-azido-2-(((9*H*-fluoren-9-ylmethoxy)carbonyl)-amino)-5-hydroxyhexanoate (10)**: *p*-Toluenesulfonic acid (2.26 g, 11.88 mmol) was added to a solution of **8**[12] (2.04 g, 5.92 mmol) in CH_3CN (30.0 mL) at 0 °C. The mixture was stirred at 0 °C for 2 h and consistently checked by TLC (AcOEt/pyridine/acetic acid/water 8:2:0.5:1, *v/v/v/v*). The mixture was then allowed to reach room temperature and stirred for an additional 2 h. The reaction was quenched by the addition of aqueous NH_4OH (1 N, 250 mL). The solution was extracted with CH_2Cl_2 (3×80.0 mL), and the combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was dissolved in THF (15.0 mL), and the same volume of water was added to the solution. Solid $NaHCO_3$ (995 mg, 11.84 mmol) and FmocOSu (2.40 g, 7.10 mmol), dissolved in the minimum volume of THF, were added to the mixture stirred at ambient temperature. After 3 h, THF was evaporated and replaced by AcOEt. The solution was washed with water and $KHSO_4$, (1 N), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH 98:2, *v/v*) to yield pure **10** (2.10 g, 76% for 2 steps) as a colourless oil. HPLC: t_R = 13.23 min (linear gradient, 30–100% B, 20 min); $[\alpha]_D = +7.3$ (c = 1.7, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ = 7.77 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.32 (m, 2H), 5.59 (bd, J = 7.8 Hz, 1H), 4.40 (d, J = 6.9 Hz, 2H), 4.34–4.30 (m, 1H), 4.22 (t, J = 6.9 Hz, 1H), 3.83–3.77 (m, 1H), 3.37–3.20 (m, 2H), 2.87 (br s, 1H), 2.07–1.97 (m, 1H), 1.78–1.68 (m, 1H), 1.62–1.52 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.3 (C), 156.1 (C), 143.8 (C), 143.7 (C), 141.3 (2C), 127.7 (2CH), 127.0 (2CH), 125.0 (2CH), 120.0 (2CH), 82.5 (C), 70.4 (CH), 67.0 (CH_2), 57.0 (CH_2), 53.8 (CH), 47.2 (CH), 29.6 (2CH_2), 28.0 (3CH_3).

General galactosylation procedure with a tetrapivaloylated donor. Procedure A: Compound **10** (1.0 equiv) and the required glycosyl donor (1.5 equiv) were placed in an argon-filled, round-bottom flask and dissolved in dry CH_2Cl_2 to yield an approximately 0.1 M solution of acceptor. Powdered 4 Å molecular sieve was added, and the suspension was stirred for 30 min. The mixture was

protected from light, and silver silicate (5.0 equiv) was added. The reaction was stirred for 8 or 16 h at room temperature and monitored by RP-HPLC. The dark-brown suspension was filtered through a plug of Celite®. The solvent was evaporated to yield a brownish residue, which was purified by flash column chromatography (Et_2O/pentane 3:7, *v/v*).

Procedure B: the required glycosylated building block was dissolved in CH_2Cl_2 to give an approximately 0.1 M solution. An equal volume of TFA was added, and the resulting solution was stirred for 2 h at ambient temperature. Following addition of a large volume of hexane, the solvents were co-evaporated to afford the glycosylated building blocks ready for use in SPPS.

***tert*-Butyl (2*S*,5*R*)-6-azido-2-(((9*H*-fluoren-9-ylmethoxy)carbonyl)-amino)-5-*O*-(2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl)hexanoate: Fmoc-(GalPiv$_4$)Hyl-O*t*Bu (13)**: Galactosylated building block **13** was prepared according to procedure A. Purification of the crude product gave **13** (yield = 850 mg, 83%) as a white foam. HPLC: t_R = 18.56 min (linear gradient, 50–100% B, 20 min); $[\alpha]_D$ = +3.1 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.77 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.31 (dt, *J* = 7.5, 1.3 Hz, 2H), 5.39 (d, *J* = 2.8 Hz, 1H), 5.36 (br d, *J* = 7.9 Hz, 1H), 5.23 (dd, *J* = 10.4, 7.7 Hz, 1H), 5.09 (dd, *J* = 10.4, 3.1 Hz, 1H), 4.69 (d, *J* = 7.9 Hz, 1H), 4.42 (dd, *J* = 10.6, 7.3 Hz, 1H), 4.36–4.29 (m, 1H), 4.24–4.13 (m, 3H), 4.06–3.94 (m, 2H), 3.80 (m, 1H), 3.82–3.73 (m, 1H), 3.45 (dd, *J* = 13.0, 4.2 Hz, 1H), 3.25 (dd, *J* = 13.0, 4.2 Hz, 1H), 1.91–1.80 (m, 1H), 1.70–1.55 (m, 3H), 1.48 (s, 9H), 1.26 (s, 9H), 1.17 (s, 9H), 1.13 (s, 9H), 1.11 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.8 (C), 177.3 (C), 176.9 (C), 176.3 (C), 171.0 (C), 155.8 (C), 143.8 (C), 143.7 (C), 141.3 (2C), 127.7 (2CH), 127.1 (2CH), 125.0 (2CH), 120.0 (2CH), 100.5 (CH), 82.6 (C), 78.1 (CH), 71.0 (2CH), 69.0 (CH), 67.1 (CH_2), 66.7 (CH), 61.3 (CH_2), 53.8 (CH), 53.7 (CH_2), 47.1 (CH), 39.0 (2C), 38.7 (2C), 28.6 (CH_2), 28.0 (3CH_3), 27.4 (CH_2), 27.1 (12CH_3); elemental analysis calcd (%) for $C_{51}H_{72}N_4O_{14}$: C 63.47, H 7.52, N 5.81; found: C 63.10, H 7.69, N 5.36.

(2*S*,5*R*)-6-azido-2-(((9*H*-fluoren-9-ylmethoxy)carbony)amino)-5-*O*-(2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl)hexanoate: Fmoc-(GalPiv$_4$)Hyl-OH (14): The *N*-Fmoc protected derivative **14** was obtained according to procedure B (yield = 565 mg, 100%) as a white foam. HPLC: t_R = 16.51 min (linear gradient, 50–100% B, 20 min); $[\alpha]_D$ = +5.0 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (m, 2H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.31 (dt, *J* = 7.3, 1.1 Hz, 2H), 5.39 (d, *J* = 3.3 Hz, 1H), 5.37 (m, 1H), 5.23 (dd, *J* = 10.4, 7.7 Hz, 1H), 5.10 (dd, *J* = 10.4, 3.1 Hz, 1H), 4.68 (d, *J* = 7.9 Hz, 1H), 4.48–4.34 (m, 3H), 4.24–4.15 (m, 2H), 4.06–3.94 (m, 2H), 3.78 (m, 1H), 3.49–3.44 (m, 1H), 3.29–3.24 (m, 1H), 2.10–1.91 (m, 1H), 1.78–1.60 (m, 3H), 1.26 (s, 9H), 1.18 (s, 9H), 1.13 (s, 9H), 1.11 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.9 (C), 177.4 (C), 177.0 (C), 176.5 (C), 175.7 (C), 156.0 (C), 143.7 (C), 143.6 (C), 141.3 (2C), 127.8 (2CH), 127.1 (2CH), 125.0 (2CH), 120.0 (2CH), 100.7 (CH), 78.2 (CH), 71.0 (2CH), 69.0 (CH), 67.2 (CH_2), 66.7 (CH), 61.4 (CH_2), 53.8 (CH_2), 53.7 (CH), 47.1 (CH), 39.0 (2C), 38.7 (2C), 28.1 (CH_2), 27.9 (CH_2), 27.1 (12CH_3); elemental analysis calcd (%) for $C_{47}H_{64}N_4O_{14}$: C 62.10, H 7.10, N 6.16; found: C 61.84, H 7.22, N 5.72.

***tert*-Butyl (2*S*,5*R*)-6-azido-2-(((9*H*-fluoren-9-ylmethoxy)carbonyl)-amino)-5-*O*-(2,3,4,6-tetra-*O*-pivaloyl-β-D-glucopyranosyl)hexanoate: Fmoc-(GlcPiv$_4$)Hyl-O*t*Bu (15)**: Galactosylated building block **15** was prepared according to procedure B. Purification of the crude product gave **15** (yield = 340 mg, 76%) as a white foam. HPLC: t_R = 18.87 min (linear gradient, 50–100% B, 20 min); $[\alpha]_D$ = +6.7 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.75 (d, *J* = 7.4 Hz, 2H), 7.59 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (dt, *J* = 7.4, 1.0 Hz, 2H), 5.39 (br d, *J* = 7.7 Hz, 1H), 5.30 (t, *J* = 9.5 Hz, 1H), 5.11 (t, *J* = 9.9 Hz, 1H), 5.01 (dd, *J* = 9.5, 7.9 Hz, 1H), 4.66 (d, *J* = 7.9 Hz, 1H), 4.42 (dd, *J* = 10.4, 7.1 Hz, 1H), 4.36–4.30 (m, 1H), 4.27–4.19 (m, 3H), 4.00 (dd, *J* = 12.2, 5.5 Hz, 1H), 3.78 (m, 1H), 3.72–3.68 (m, 1H), 3.45 (dd, *J* = 12.8, 4.2 Hz, 1H), 3.25 (dd, *J* = 12.8, 4.4 Hz, 1H), 1.88–1.77 (m, 1H), 1.70–1.53 (m, 3H), 1.47 (s, 9H), 1.21 (s, 9H), 1.14 (s, 9H), 1.12 (s, 9H), 1.10 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.9 (C), 177.1 (C), 176.3 (C), 176.1 (C), 170.9 (C), 155.8 (C), 143.8 (C), 143.7 (C), 141.3 (2C), 127.7 (2CH), 127.1 (2CH), 125.0 (2CH), 120.0 (2CH), 100.1 (CH), 82.6 (C), 77.9 (CH), 72.2 (2CH), 71.4 (CH), 67.9 (CH), 67.1 (CH_2), 61.7 (CH_2), 53.7 (CH), 53.7 (CH_2), 47.1 (CH), 38.8 (C), 38.7 (C), 38.6 (2C), 28.5 (2CH_2), 28.0 (3CH_3), 27.1 (12CH_3); elemental analysis calcd (%) for $C_{51}H_{72}N_4O_{14}$: C 63.47, H 7.52, N 5.81; found: C 62.64, H 7.54, N 5.91.

(2*S*,5*R*)-6-Azido-2-(((9*H*-fluoren-9-ylmethoxy)carbony)amino)-5-*O*-(2,3,4,6-tetra-*O*-pivaloyl-β-D-glucopyranosyl)hexanoate: Fmoc-(GlcPiv$_4$)Hyl-OH (16): The *N*-Fmoc-protected derivative **16** was obtained according to procedure B (yield = 565 mg, 100%) as a colourless oil. HPLC: t_R = 17.02 min (linear gradient, 50–100% B, 20 min); $[\alpha]_D$ = +6.9 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.75 (d, *J* = 7.5 Hz, 2H), 7.59–7.55 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 5.55 (m, 1H), 5.29 (t, *J* = 9.3 Hz, 1H), 5.08 (t, *J* = 9.9 Hz, 1H), 4.98 (t, *J* = 8.6 Hz, 1H), 4.58 (m, 1H), 4.36 (m, 2H), 4.26–4.18 (m, 3H), 3.97–3.94 (m, 1H), 3.76–3.61 (m, 1H), 3.47–3.38 (m, 1H), 3.26–3.15 (m, 1H), 1.96–1.83 (m, 1H), 1.71–1.55 (m, 3H), 1.20 (s, 9H), 1.14 (s, 9H), 1.10 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.1 (C), 177.2 (C), 176.5 (C), 176.3 (C), 175.1 (C), 156.6 (C), 143.8 (C), 143.6 (C), 141.2 (2C), 127.8 (2CH), 127.1 (2CH), 125.1 (2CH), 120.0 (2CH), 100.4 (CH), 82.6 (C), 78.2 (CH), 72.2 (2CH), 71.5 (CH), 67.8 (CH), 67.3 (CH_2), 61.7 (CH_2), 53.6 (CH), 53.6 (CH_2), 47.0 (CH), 38.8 (C), 38.7 (C), 38.6 (2C), 29.7 (CH_2), 27.8 (CH_2), 27.1 (6 CH_3), 27.0 (6 CH_3); elemental analysis calcd (%) for $C_{47}H_{64}N_4O_{14}$: C 62.10, H 7.10, N 6.16; found: C 62.31, H 7.28, N 5.98.

General procedure for SPPS. Procedure C: Glycopeptides **1**–**6** and **21**–**23** were synthesised on Wang resin.[20] by using a home-made, semiautomatic peptide synthesiser.[21] For each coupling step, the reactants were introduced manually as a solution in dry DMF (2.0 mL). N^{α}-Fmoc amino acids (5.0 equiv) with standard side-chain-protecting groups were coupled twice by using (1-benzotriazolyl)oxy tris(dimethylamino) phosphonium hexafluorophosphate (BOP; 5.0 equiv), 1-hydroxybenzotriazole (HOBt; 5.0 equiv) and diisopropylethylamine (DIEA, 10.0 equiv) in dry DMF for 20 min. Glycosylated building blocks **14**, **16** and **17**–**25** (2.0 equiv) were coupled twice by using BOP (2.0 equiv), HOBt (2.0 equiv) and DIEA (4.0 equiv) in dry DMF for 60 min (1st coupling) and 20 min (2nd coupling). The washing of the resin, as well as Fmoc deprotection (by using a freshly prepared solution of 20% piperidine in DMF), was performed automatically. The coupling and deprotection steps were monitored by using the Kaiser test.[29] At the end of the elongation of the peptidic chain, the resin was washed with CH_2Cl_2 and dried with Et_2O before the next step.

General procedure for azide reduction on solid support. Procedure D: The resin was placed in a syringe equipped with a frit and swallowed by the addition of THF (1.0 mL). A solution of PPh_3 (10.0 equiv) in THF/water (3:1) was added, and the suspension was gently shaken for 72 h. The resin was washed with THF and CH_2Cl_2 and dried with Et_2O before the next step.

General procedure for cleavage from the resin. Procedure E: A mixture of TFA/H_2O/TIPS/DTT (8.8:0.5:0.2:0.5; 10.0 mL) was added to the resin. The mixture was gently shaken for 2.5 h, and the resulting solution was flushed through a frit in cold Et_2O. The precipi-

tate was recovered by centrifugation, dissolved in a mixture of AcOH and H_2O and freeze-dried.

General procedure for deprotection of the glycosyl moiety. Procedure F: The glycopeptide (white powder) was placed in a round-bottomed flask and dissolved in a freshly prepared solution of NaOMe in MeOH (40.0 mM). The deprotection was monitored by analytical C_{18} RP-HPLC. After full deprotection of the glycosyl moiety, the solution was neutralised by dropwise addition of AcOH, and MeOH was removed in vacuo.

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hyl)-Gly-Glu-Gln-Gly-Pro-Lys270 (1): The synthesis of **1** was performed with building block **14** on resin (60 μmol) by using the general procedures C–F. The purity of the crude peptide was 72% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **1** (yield = 59 mg, 59% and >99% purity). HPLC: t_R = 11.44 min (linear gradient, 5–65% B, 20 min); calcd mass: 1666.75; found: 1667.11 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hnl(6-N3))-Gly-Glu-Gln-Gly-Pro-Lys270 (2): The synthesis of **2** was performed with building block **14** on resin (60 μmol) according to procedures C, E and F. The purity of the crude peptide was 70% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure 2 (yield = 60 mg, 59% and >99% purity). HPLC: t_R = 11.95 min (linear gradient, 5–65% B, 20 min); calcd mass: 1691.75; found: 1692.92 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hnl(6-OH))-Gly-Glu-Gln-Gly-Pro-Lys270 (3): The synthesis of **3** was performed with building block **14**[13] on resin (60 μmol) by using procedures C, D and F. The purity of the crude peptide was 67% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure 3 (yield = 58 mg, 58% and >99% purity). HPLC: t_R = 11.74 min (linear gradient, 5–65% B, 20 min); calcd mass: 1666.74; found: 1667.01 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5S)-Hyl)-Gly-Glu-Gln-Gly-Pro-Lys270 (4): The synthesis of **4** was performed with building block **14**[13]on resin (60 μmol) by using procedures C–F. The purity of the crude peptide was 83% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **4** (yield = 58 mg, 58% and 95% purity). HPLC: t_R = 11.93 min (linear gradient, 5–65% B, 20 min); calcd mass: 1665.75; found: 1667.84 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-6-Hnl[(5S)-NH2])-Gly-Glu-Gln-Gly-Pro-Lys270 (5): The synthesis of **5** was performed with building block **14**[13] on resin (60 μmol) according to procedures C–F. After reduction of the azido moiety and TFA treatment, the crude pivaloylated glycopeptide ([M+H$^+$] = 2001.98) was contaminated by a major impurity **27** ([M+H$^+$] = 2262.26, HPLC: t_R = 15.53 min (linear gradient, 20–80% B, 20 min), see Supporting Information) and was purified by HPLC prior to removal of pivaloyl ester protecting groups. Final purification by semipreparative C_{18} RP-HPLC gave pure **5** (yield = 28.5 mg, 29% and >99% purity). HPLC: t_R = 10.13 min (linear gradient, 5–65% B, 20 min); calcd mass: 1665.75; found: 1666.70 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hyl(5-Me))-Gly-Glu-Gln-Gly-Pro-Lys270 (6): The synthesis of **6** was performed with building block **14**[13] on resin (60 μmol) by using procedures C–F. The purity of the crude peptide was 70% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **6** (yield = 57 mg, 57% and >99% purity). HPLC: t_R = 11.21 min (linear gradient, 5–65% B, 20 min); calcd mass: 1679.78; found: 1680.17 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Glc-5-Hyl)-Gly-Glu-Gln-Gly-Pro-Lys270 (21): The synthesis of **21** was performed with building block 16 on resin (80 μmol) according to procedures C–F. The purity of the crude peptide was 75% (determined by C_{18} RP-HPLC). Purification by semi-preparative C_{18} RP-HPLC gave pure **21** (73 mg, 55% and 95% purity). HPLC: t_R 12.60 (linear gradient, 5–65% B, 20 min); calcd mass: MM = 1665.75; found: [M+H$^+$] = 1667.28.

Gly256-Glu-Pro-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hyl)-Gly-Glu-Gln-Gly-Pro-Lys270 (22): The synthesis of **22** was performed with building block **14** on resin (80 μmol) by using procedures C–F. The purity of the crude peptide was 80% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **22** (yield = 74 mg, 56% and 99% purity). HPLC: t_R = 12.18 min (linear gradient, 5–65% B, 20 min); calcd mass: 1649.75; found: 1651.18 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal-(5R)-Hyl)-Gly-Asp-Gln-Gly-Pro-Lys270 (23): The synthesis of **23** was performed with building block **14** on resin (60 μmol) by using procedures C–F. The purity of the crude peptide was 63% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **23** (yield = 57 mg, 58% and >99% purity). HPLC: t_R = 11.71 min (linear gradient, 5–65% B, 20 min); calcd mass: 1651.73; found: 1653.66 [M+H$^+$].

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys270 (24): The synthesis of **24** was performed with commercial amino acids on resin (25 μmol) by using procedures C and E. The purity of the crude peptide was 85% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **24** (yield = 35 mg, 95% and 98% purity). HPLC: t_R = 11.86 min (linear gradient, 5–65% B, 20 min); calcd mass: 1487.61; found: 1488.85 [M+H$^+$].

Gly256-Glu-Pro-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys270 (25): The synthesis of **25** was performed with commercial amino acids on resin (25 μmol) by using procedures C and E. The purity of the crude peptide was 86% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **25** (yield = 35 mg, 95% and >99% purity). HPLC: t_R = 12.08 min (linear gradient, 5–65% B, 20 min); calcd mass: 1471.61.; found: [M+H$^+$] = 1473.08.

Gly256-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-(Gal(Piv4)-(5R)-Hyl)-Gly-Glu-Gln-Gly-Pro-Lys270 (26): The synthesis of **26** was performed with building block **14** on resin (60 μmol) by using procedures C-E. The purity of the crude peptide was 86% (determined by C_{18} RP-HPLC). Purification by semipreparative C_{18} RP-HPLC gave pure **26** (yield = 88 mg, 73% and >99% purity). HPLC: t_R = 13.81 min (linear gradient, 20–80% B, 20 min); calcd mass: 2002.22; found: 2002.65 [M+H$^+$].

Generation of CII-specific T-cell hybridomas and T-cell clones: The three anti-CII CD4$^+$ T-cell hybridomas required for this study were isolated by Chiocchia and colleagues[9] The T-cell hybridomas were derived by fusion of lymph-node cells from CII-primed, DBA/1 (H-2^q) mice (immunised with bCII emulsified in complete Freund adjuvant (CFA) and the mutant BW5147 thymoma cells (H-2^k). The anti-CII T-cell clone A9.2 was isolated by Doncarli and colleagues[10] from the lymph nodes of CII-immunized DBA/1 mice.

Determination of T-cell reactivity: T-cell responses were assessed by means of proliferation measurement for the A9.2 clone and quantification of IL-2 secretion for T hybridomas. The antigen-presenting cells (APC) used were either DBA/1-irradiated spleen cells (5×10^5 cells per well) or paraformaldehyde-fixed M12.C10 cells (10^5 cells per well), an I-A^{q+} B lymphoma that we generated.[30] In all tests, T-cell clones (3×10^4 cells per well) or T hybridomas (10^5 cells

per well) were cocultured in triplicate with APC in the presence of varying concentrations of glycopeptides in culture medium (total volume 200 μL). The A9.2 cell cultures were incubated at 37 °C under 5% CO_2 atmosphere for 3 days. [^{3}H]thymidine (0.5 μCi per well) was added during the last 16 h of culture, and the radioactivity incorporated by the cells was determined by liquid scintillation counting. Regarding the T hybridoma cultures, supernatants were collected after 24 h of incubation and frozen at −20 °C. Thawed supernatants were tested for their ability to support CTLL-2 proliferation by following the procedure of [^{3}H]thymidine incorporation described above. The results are expressed as a stimulation index (ratio of peptide-stimulated to medium-treated cocultures).

Acknowledgements

J.M. thanks the CNRS and NeoMPS for a predoctoral fellowship, as well as the Fondation pour la Recherche Médicale for its support. This research was supported in part by the CNRS and INSERM.

Keywords: glycopeptides · hydroxylysine · immunology · rheumatoid arthritis · solid-phase synthesis

[1] Statistics from the WHO Statistical Information System (WHOSIS), http://www3.who.int/whosis/menu.cfm
[2] M. Feldman, F. M. Brennan, R. V. Maini, *Cell* **1996**, *85*, 307 – 310.
[3] For a review, see: R. Holmdhal in *Rheumatoid Arthritis* (Eds.:J. S. Smolen, J. R. Kalden, R. N. Maini), Springer, Berlin, **1992**, pp. 180 – 201.
[4] a) D. E. Trentham, A. S. Townes, A. H. Kang, *J. Exp. Med.* **1977**, *146*, 857 – 868; b) J. S. Courtenay, M. J. Dallman, A. D. Dayan, A. Martin, B. Mosedale, *Nature* **1980**, *283*, 666 – 667.
[5] For a review, see: R. Holmdahl, M. Andersson, T. J. Goldschmidt, K. Gustafsson, L. Jansson, J. A. Mo, *Immunol. Rev.* **1990**, *118*, 193 – 232.
[6] a) L. K. Myers, K. Terato, J. M. Seyer, J. M. Stuart, A. H. Kang, *J. Immunol.* **1992**, *149*, 1439 – 1443; b) E. Michaëlsson, M. Andersson, A. Engström, R. Holmdahl, *Eur. J. Immunol.* **1992**, *22*, 1819 – 1825.
[7] For a review, see: R. Holmdahl, E. C. Andersson, C. B. Andersen, A. Svejgaard, L. Fugger, *Immunol. Rev.* **1999**, *169*, 161 – 173.
[8] a) E. Michaëlsson, V. Malmström, S. Reis, A. Engström, H. Burkhardt, R. Holmdahl, *J. Exp. Med.* **1994**, *180*, 745 – 749; b) P. Kjellén, U. Brunsberg, J. Broddefalk, B. Hansen, M. Vestberg, I. Ivarsson, A. Engström, A. Svejgaard, J. Kihlberg, R. Holmdahl, *Eur. J. Immunol.* **1998**, *28*, 755 – 767; c) A. Corthay, J. Bäcklund, J. Broddefalk, E. Michaëlsson, T. J. Goldschimdt, J. Kihlberg, R. Holmdahl, *Eur. J. Immunol.* **1998**, *28*, 2580 – 2590; d) J. Broddefalk, J. Bäcklund, F. Almqvist, M. Johansson, R. Holmdahl, J. Kihlberg, *J. Am. Chem. Soc.* **1998**, *120*, 7676 – 7683; e) B. Holm, J. Bäcklund, M. A. F. Recio, R. Holmdahl, J. Kihlberg, *ChemBioChem* **2002**, *3*, 1209 – 1222; f) J. Bäcklund, S. Carlsen, T. Höger, B. Holm, L. Fugger, J. Kihlberg, H. Burkhardt, R. Holmdahl, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 9960 – 9965.
[9] G. Chiocchia, B. Manoury-Schwartz, M. C. Boissier, H. Gahery, P. N. Marche, C. Fournier, *Eur. J. Immunol.* **1994**, *24*, 2775 – 2783.
[10] A. Doncarli, G. Chiocchia, L. M. Stasiuk, D. Herbage, M. M. Boutillon, C. Fournier, O. Abehsira-Amar, *Eur. J. Immunol.* **1999**, *29*, 3636 – 3642.
[11] a) J. Sloan-Lancaster, P. M. Allen, *Annu. Rev. Immunol.* **1996**, *14*, 1 – 27; b) S. Brocke, K. Gijbel, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchell, T. Veromaa, A. Waisman, A. Gaur, P. Conlon, N. Ling, P. J. Fairchild, D. C. Wraith, A. O'Garra, C. G. Fathman, L. Steinman, *Nature* **1996**, *379*, 343 – 346.
[12] J. Marin, C. Didierjean, A. Aubry, J.-P. Briand, G. Guichard, *J. Org. Chem.* **2002**, *67*, 8440 – 8449.
[13] a) For examples of selective deprotection with HCl, see: R. A. August, J. A. Khan, C. M. Moody, D. W. Young, *J. Chem. Soc. Perkin Trans. 1* **1995**, 507 – 514; F. S. Gibson, S. C. Bergmeier, H. Rapoport, *J. Org. Chem.* **1994**, *59*, 3216 – 3218; M. S. Stanley, *J. Org. Chem.* **1992**, *57*, 6421 – 6430; b) for an example of selective deprotection with sulfuric acid, see: L. S. Lin, T. Lanza, Jr., S. E. de Laszlo, Q. Truong, T. Kamenecka, W. K. Hagmann, *Tetrahedron Lett.* **2000**, *41*, 7013 – 7016; c) for examples of selective deprotection with PTSA, see: J. E. Baldwin, R. M. Adlington, C. R. A. Godfrey, D. W. Gollins, C. J. Schofield, *Tetrahedron* **1991**, *47*, 5835 – 5840; J. Goodacre, R. J. Ponsdorf, I. Stirling, *Tetrahedron Lett.* **1975**, *16*, 3609 – 3612.
[14] J. Marin, A. Violette, J. -P. Briand, G. Guichard, *Eur. J. Org. Chem.* **2004**, 3027 – 3039.
[15] While this manuscript was in preparation, Kihlberg and co-workers reported similar difficulties and did not succeed in galactosylating a related N-Fmoc-protected "azido-hydroxylysine" glycosyl acceptor with peracetylated galactosyl bromide and silver silicate as a promoter. B. M. Syed, T. Gustafsson, J. Kihlberg, *Tetrahedron* **2004**, *60*, 5571 – 5575.
[16] In contrast, Fmoc-Hyl(*Z*)-OAll and Fmoc-Hyl(Boc)-OBn were galactosylated in 82% and 45% yield, respectively, by using peracetylated galactosyl bromide: a) B. Holm, J. Broddefalk, S. Flodell, E. Wellner, J. Kihlberg, *Tetrahedron* **2000**, *56*, 1579 – 1586; b) M. Cudic, J. L. Lauer-Fields, G. B. Fields, *J. Pept. Res.* **2005**, *65*, 272 – 283.
[17] W. Koenigs, E. Knorr, *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 957 – 981.
[18] R. R. Schmidt, M. Hoffmann, *Angew. Chem.* **1983**, *95*, 417 – 418; *Angew. Chem. Int. Ed. Engl.* **1983**, *22*, 406 – 407.
[19] *Fmoc Solid Phase Peptide Synthesis—A Practical Approach* (Ed.: W. S. Chan, P. D White), Oxford University Press, Oxford, **2000**.
[20] S. S. Wang, *J. Am. Chem. Soc.* **1973**, *95*, 1328.
[21] J. Neimark, J. P. Briand, *Pept. Res.* **1993**, *6*, 219.
[22] The presence of the triarylphosphine moiety in **27** was initially deduced from mass-spectrometry measurements. The identity of the triphenyl phosphonium peptide side product was further supported by detailed ^{1}H, ^{31}P and HMQC ^{1}H-^{31}P NMR experiments (see Supporting Information), in which the phosphorus displays a chemical shift consistent with known alkylaminotriphenylphosphonium salts. The aminotriphenylphosphonium salt glycopeptide **27** was purified by RP-HPLC and found to be extremely stable as a solid (lyophilised powder) and in solution (water and [D_6]DMSO). The formation of an intramolecular salt (for another example, see M. Brewer, D. H. Rich, *Org. Lett.* **2001**, *3*, 945 – 948) with the side chain of Glu266 might account for the observed stability of **27**.
[23] P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson, R. A. Dwek, *Science* **2001**, *291*, 2370 – 2376.
[24] D. Dzhambazov, M. Holmdahl, Y. Hisakata, S. Lu, M. Vestberg, B. Holm, O. Johnell, J. Kihlberg, R. Holmdahl, *Eur. J. Immunol.* **2005**, *35*, 357 – 366.
[25] J. Bäcklund, A. Treschow, R. Bockermann, B. Holm, L. Holm, S. Issazadeh-Navikas, J. Kihlberg, R. Holmdahl, *Eur. J. Immunol.* **2002**, *32*, 3776 – 3784.
[26] L. Steinman, *Science* **2004**, *305*, 212 – 216.
[27] a) H. Kunz, W. Sager, D. Schanzenbach, M. Decker, *Liebigs Ann. Chem.* **1991**, *7*, 649 – 654; b) M. Osswald, U. Lang, S. Friedrich-Bochnitschek, W. Pfrengle, H. Kunz, *Z. Naturforsch. B* **2003**, *58*, 764 – 774.
[28] H. Paulsen, O. Lockoff, *Chem. Ber.* **1981**, **114**, 3102 – 3114.
[29] E. Kaiser, R. L. Colescott, C. D. Bosinger, P. Cook, *Anal. Biochem.* **1970**, *34*, 595 – 598.
[30] B. Manoury-Schwartz, G. Chiocchia, V. Lotteau, C. Fournier, *Int. Immunol.* **1997**, *9*, 581 – 589.

Received: February 21, 2005
Published online on August 23, 2005

